ALPHA- FETOPROTEIN EXPRESSION IN HEPATOCELLULAR CARCINOMA

DISSERTATION

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G.S.THIRIVENI BALAJJI
DEPARTMENT OF PATHOLOGY
PSG INSTITUTE OF MEDICAL SCIENCES & RESEARCH
PEELAMEDU, COIMBATORE – 641 004.
TAMILNADU, INDIA
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CERTIFICATE

This is to certify that the dissertation work entitled "**ALPHA FETO – PROTEIN EXPRESSION IN HEPATOCELLULAR CARCINOMA**" submitted by **Dr.G.S.Thiriveni Balajji** is the work done by her during the period of study in this department from June 2003 to February 2006. This work has been done under my direct supervision and guidance.

Dr.S.Ramalingam
Principal
PSG IMS & R
Coimbatore

Dr. K.R. Bagyalakshmi
Professor&Head of the Department
Department of Pathology
PSG IMS & R
Coimbatore.

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INTRODUCTION

On a global basis, primary liver cancer which is almost entirely hepatocellular carcinoma constitutes approximately 5.6% of cancers and in some populations is the most common cancer[1,2]. The largest numbers of cases are found in Asia (76% of all hepatocellular carcinoma), followed by Africa. Annual incidence rates for hepatocellular carcinoma are below 5 cases per 1,00,000 population in North and South America, North and Central Europe and Australia with intermediate rates of upto 15 cases per 100,000 in countries bordering the Mediterranean. The highest annual incidence rates are found in Korea, Taiwan, Mozambique and Southeast China, approaching 36 per 100,000. Within each geographic area (low or high incidence), blacks have attack rates approximately threefold higher than Caucasians.

In India, it is estimated that there are approximately 2 – 2.5 million cases of cancer at any given point of time, with around 7,00,000 new cases being detected each year. Nearly half of these cases die each year[3]. The incidence of liver malignancy is about 5.6 percent[4] and among the top three causes of death from cancer are included the cancer of lung (17.8% of all deaths), stomach (10.4%) and liver (8.8%)[4]. In India, the National Cancer Registry Programme of the ICMR provides data on incidence from five population based registries. The age standardized cancer incidence at Mumbai in India for liver, was 4.9 per 1,00,000 in males and 2.5 per 1,00,000 in females[3].

Worldwide, there is a clear predominance of males, ranging from 1.5:1 in countries with a low incidence of hepatocellular carcinoma to approximately 3:1 in populations with a high frequency. More than 85% of cases of hepatocellular carcinoma occur in countries with high rates of chronic hepatitis B viral infection incidence.[5]

There is strong evidence of a pathogenetic role of hepatotropic viruses in the development of liver cell carcinoma, not only through the causation of cirrhosis but also in non - cirrhotic livers[6,7]. This is true for both hepatitis B and hepatitis C viruses and for both adult and pediatric patients[8].

In one series from Japan, the development rate of hepatocellular carcinomas after a 15-year observation period was 27% in hepatitis B and 75% in hepatitis C[9]. The incidence of hepatitis B and / or hepatitis C surface antigenemia in patients with liver cell carcinoma is over 90%, both in the United States and in other countries[10,11] In patients with hepatitis, increased proliferative indices and high alpha - fetoprotein serum value represent risk markers for liver cell carcinoma[12,13].

Serum alpha - fetoprotein levels remain the most useful marker for hepatocellular carcinoma . Serum alpha - fetoprotein levels are elevated (>10 to 20 ng/ml) in about 70% to 80% of patients (specificity, 90%) with hepatocellular carcinoma. It is frequently detectable at levels greater than 200 ng/ml in HBV related and HCV related cases ($\simeq$ 76%); however, tumors arising in the context of

alcohol related cirrhosis (65%), those found in the non cirrhotic liver (33%), and those less than 2 cm in diameter (25%) less often show elevations of this magnitude, and serum alpha - fetoprotein may even be undetectable in all these cases[14].

Guided fine needle aspiration biopsy is increasingly being recognized as an excellent diagnostic method for detecting hepatic malignancy[15]. Fine needle aspiration has several advantages over thick needle biopsies :

a. Multiple aspirations can safely be performed to ensure an adequate specimen.
b. Specimen adequacy can be checked and immediate interpretation of the material is possible.
c. Left lobe, porta hepatis can be reached and can be performed in patients with ascites, portal hypertension or obstructive jaundice.
d. Implantation of malignant cells along the needle tract appears to be less common than with thick needle biopsy.

Therefore, for any mass or masses in the liver suspected to be malignant, guided fine needle aspiration cytology is the method of choice[16]. The sensitivity of guided fine needle aspiration cytology for a diagnosis of hepatic malignancy ranges from 90% to 96% with a specificity of 90% to 100%[17]. The application of immunocytochemical technique further enhances diagnostic accuracy in the typing of hepatic cancers.

Immunocytochemistry on fine needle aspiration smears is both a science requiring specialized training and an art demanding dedication and nurture. Immunocytochemistry has the potential of transforming surgical pathology from a subjective “art” to an objective “science” based on the way cells can be recognized by microscopic methods. Given the choice of vast array of antibodies that can be used in immunocytochemistry, the problem lies in choosing wisely.

High serum levels of alpha - fetoprotein provide strong support for the diagnosis of hepatocellular carcinoma. Therefore serologic tests and a detailed clinical history are important, especially in difficult cases.

12% to 62% of hepatocellular carcinomas are positive for alpha-fetoprotein in immunocytochemistry[17]. Hence, a positive immunocytochemistry result strongly supports the diagnosis of hepatocellular carcinoma, but a negative result does not exclude hepatocellular carcinoma[19].

The purpose of this study is to determine whether alpha - fetoprotein could be used to aid in the diagnosis of hepatocellular carcinoma and also to evaluate the distribution of alpha - fetoprotein in hepatocellular carcinomas of varying differentiation.

AIMS AND OBJECTIVES

1. **To assess the percentage positivity of cytological expression of alpha - fetoprotein in fine needle aspiration smears of hepatocellular carcinoma .**

2. To correlate the percentage positivity of cytological alpha - fetoprotein expression and serum alpha - fetoprotein level with the differentiation of hepatocellular carcinoma.

3. To correlate the incidence of hepatocellular carcinoma with hepatitis B and hepatitis C viral infections.

REVIEW OF LITERATURE

Hepatocellular carcinoma, a unique human neoplasm, has interested many in several fields of biological and health sciences. This cancer is credited as the first that could be largely eliminated by a safe anti-viral vaccine, the hepatitis B vaccine. It is also the first cancer for which a reliable diagnostic tumour marker – alpha - fetoprotein was identified. Also studies on this tumour in humans and animals have provided a large body of information on causation and stepwise evolution of cancers. Being a common and rapidly fatal tumour, mainly affecting males in the more populous developing countries, hepatocellular carcinoma may well be the commonest cancer of the human males[20].

GLOBAL INCIDENCE AND DISTRIBUTION:

Hepatocellular carcinoma constitutes about 85% of primary liver cancer recorded in cancer data banks and records. It is the fifth most common neoplasm in the world and the third most common cause of cancer related death [(21)]

Incidence and mortality of five most common cancers world wide, 2000[21].

Site	Number of Cases (Incidence%)	Number of deaths (Mortality %)
Lung	1238.9 (12.3%)	1103.1 (17.7%)
Breast	1050.3 (10%)	373 (6%)
Colorectum	944.7 (9.4%)	492.4 (7.9)
Stomach	876.3 (8.7%)	646.6 (10.4%)
Liver	564.3 (5.6%)	548.6 (8.8%)
Total	10,055.6 (100%)	6208.7 (100%)

Globally, around 5,00,000 new cases are diagnosed yearly, with an age adjusted world wide incidence of 5.6 to 14.9 per 1,00,000 population.. It accounts for 5.6% of all human cancers with incidence greater in males (7.5%) than in females (3.2%).Almost the same number die of this cancer annually[21]

PREDISPOSING AND ASSOCIATED FACTORS :

Hepatotropic viruses, both hepatitis B and hepatitis C virus are believed to be causally associated in the development of hepatocellular carcinoma.

HEPATITIS B VIRUS & HEPATOCELLULAR CARCINOMA :

HBV is a member of hepadna virus group and is a double stranded DNA virus that replicates by reverse transcription. It is a 42 nm particle comprising an electron dense core (nucleocapsid) which is 27 nm in diameter

and an outer envelope of the surface in membranous lipid derived from the host cell.

Markers of viral replication in serum include HBV DNA, the surface protein HBsAg, and a soluble antigen HBeAg which is secreted by infected hepatocytes. HBsAg is detectable by radio immunoassay or enzyme immunoassay. Enzyme immunoassay is specific and highly sensitive and is used in preference to radio immunoassay.

The antigen persists during the acute phase of the disease and sharply decreases when antibody to the surface antigen becomes detectable. The virus persists in approximately 5 – 10% of immunocompetent adults and in as many as 90% of infants infected perinatally. Persistent carriage of HBV is defined by the presence of HBs Ag in the serum for more than 6 months and it has been estimated to affect about 3,50,000,000 people worldwide[22].

A number of studies have shown a relationship between persistent or past infection with HBV and the eventual development of hepatoma especially in areas of the world where the prevalence of HBV is high. It is noteworthy that the frequency of hepatoma follows the same geographic pattern of distribution as that of persistent HBV infection. The frequency of finding

HBsAg in hepatoma patients is significantly higher than in controls living in the same region.

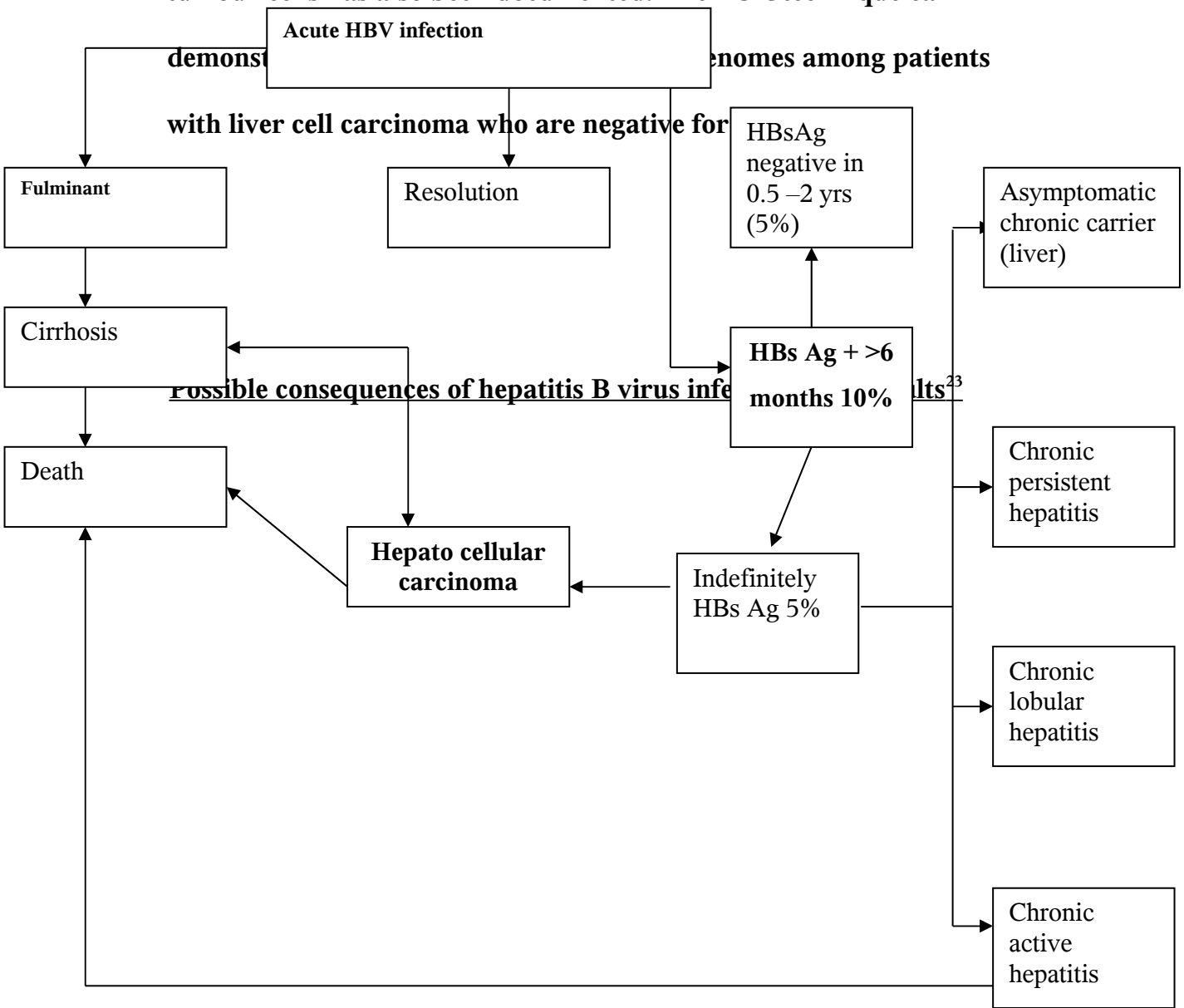
Although there are millions of HBV carriers in the world, hepatoma is relatively uncommon. This situation is not unusual in viral infections. The risk factors other than exposure to HBV which are associated with development of hepatoma are not known, but age when first infected appears to be important[22].

Once infected with HBV, males are more likely to remain persistently infected, whereas females are more likely to be transiently infected and to develop anti HBs. More males than females develop cirrhosis of the liver (by a ratio of 3:1) and an even greater proportion develop liver cancer (by a ratio of 6:1). Thus at each progressive stage of liver disease the ratio of males to females appear to double so that hepatocellular carcinoma becomes predominantly a disease of male[23].

In a prospective study by Bearley et al among 22, 707 Oriental males in Taiwan followed for an average of 6.2 years, the risk of developing hepatocellular carcinoma proved to be 217 times greater for the HBsAg carriers than for the non carriers. An overall average of one hepatoma death per 322 HBsAg carriers per year has been observed. This translates to approximately 0.3% deaths from liver cancer per year among male Oriental carriers. Thus it can be estimated that the risk

of developing primary liver cancer in an Oriental HBsAg positive carrier during a 50 years surveillance period is about 15%[23].

In a recent study involving almost 11,000 men in Taiwan, positivity for HBeAg was found to be definitely associated with an increased risk for the development of hepatocellular carcinoma. Both surface and core antigens can be detected by immunohistochemistry in the cytoplasm of the tumor cells and the non neoplastic hepatocytes in many of the cases. Integration of hepatitis virus DNA into the genome of the tumour cells has also been documented. The PCR technique can demonst enomes among patients with liver cell carcinoma who are negative for



Possible consequences of hepatitis B virus infe lts[23]

HEPATITIS C VIRUS AND HEPATOCELLULAR CARCINOMA :

HCV is an enveloped single stranded RNA virus 30 – 60 nm in size that is related to flavi viruses. ELISA can be used to detect anti HCV antibodies. Positive reactions by ELISA require confirmation by immunoblot assay. Most acute infections with HCV are asymptomatic and about 20% of acute infections cause jaundice and fulminant hepatitis. Current data suggest that about 80% of infection with HCV progress to chronicity.

Histological examination of liver biopsies from asymptomatic HCV carriers has revealed chronic active hepatitis, cirrhosis or both in upto 70%. Infection with HCV is also associated with progression to primary liver cancer.

In Japan, where the incidence of hepatocellular carcinoma has been increasing despite a decrease in the prevalence of HBs Ag, it is believed that HCV is a major risk factor[22]. Hepatocellular carcinoma, in developing countries affect young patients with chronic hepatitis B virus infection whereas in developed countries it appears in older patients related to hepatitis C virus infection[21].

Cirrhosis, liver cell dysplasia (including both large cell dysplasia and small cell dysplasia), adenomatous hyperplasia are the lesions associated with high incidence of hepatocellular carcinoma. Other etiological agents include Aflatoxin, Thorium dioxide exposure, Androgenic anabolic steroids, Progestational agents and Alpha 1 – antitrypsin deficiency[12].

Pathway of carcinogenesis :

A stepwise carcinogenesis for hepatocellular carcinoma has been proposed on the basis of gradually increasing size and cellular density among hepatocellular lesions. According to the currently used terminology, the stepwise sequence of events can be translated as follows :

Several authors have proposed a de novo pathway, a single cell or a group of hepatocytes may give rise to a focus of small hepatocellular carcinoma that will grow into a large hepatocellular carcinoma [24].

MACROSCOPIC TYPES OF HEPATOCELLULAR CARCINOMA : [(12)]

Grossly, liver cell carcinoma may present in one of the following forms.

1. Single mass - Solitary
2. Multiple Nodules - Multinodualr
3. Diffuse Lesion - Diffuse
4. Large mass in the liver - Massive form.

HISTOLOGIC GRADING OF HEPATOCELLULAR CARCINOMA :

The histologic grading to tumor differentiation is assigned by using the Edmondson grading system[(24, 25)].

Grade I

- Cells similar in size to normal hepatocytes
- Arranged in relatively thin trabeculae
- Areas containing bile are rare

Grade II

- Cells are larger than normal hepatocytes with more hyperchromatic nuclei which occupy a higher proportion of cells.
- Trabeculae are thicker.
- Acini with bile are common.

Grade III

- Hepatocytes with larger nuclei, occupying more than 50% of the cytoplasm.
- Trabeculae are still dominant, but solid areas and isolated cells may also be present.
- Giant cells common.
- Bile is rarely present.

Grade IV

- Cells with nuclei occupying most of the cytoplasm, and the cytoplasm may not be eosinophilic.
- Mostly solid areas are found.
- Bile is rarely found.
- Intravascular and intrasinusoidal growth is common.
- Includes spindle cell and small cell components.

With the above grading system, grade I cell populations may be difficult to distinguish from hepatocellular adenomas, and grade IV cell populations may be difficult to distinguish from tumors of nonhepatocellular origin[22, 23].

HEPATOCELLULAR CARCINOMA IN CYTOLOGY :

Hepatocellular carcinoma is the most common primary hepatic malignancy[19]. Fine needle aspiration cytology is still considered as a necessary procedure for diagnosing hepatocellular carcinoma.

DEFINITION OF CYTOLOGICAL CRITERIA :[25]

1. **High cellularity -** Cellular material covering 50% of atleast one slide.
2. **Increased nuclear size -** Nuclei more than twice the diameter of a normal hepatocyte nucleus.
3. **Increased N : C ratio -** Ratio more than 1:4 (Normal hepatocyte ratio 1:5)
4. **Macronucleolus -** Easily visible at x250
5. **Increased mitoses -** More than 1/20 high power field.
6. **Nuclear atypia -** Hyperchromasia and irregular nuclear membrane.
7. **Large fragments -** Microbiopsies, usually with central vessels and / or more than 100 cells.
8. **Intermediate sized clusters -** More than 10 or less than 100 cells.
9. **Small groups -** 4 – 10 cells.
10. **Widened Trabeculae -** More than 2 cells thick.

The cytologic appearance of hepatocellular carcinoma varies with the degree of differentiation. The cytologic diagnosis of hepatocellular carcinoma can be separated into two main categories, that is, low grade (well-differentiated) and high grade tumors (moderate and poorly differentiated)[19].

Cytological appearance of low grade hepatocellular carcinoma (well differentiated) :[19]

1. Low power smear pattern with smooth-edged clusters and thickened trabeculae with peripherally wrapped endothelial cells (virtually pathagnomonic)
2. Low power smear pattern with more than focal, loosely cohesive sheets of hepatocytes with transgressing vessels (highly suspicious finding).
3. Monotonous uniform hepatocytic cell population with subtle malignant features.
4. Acinar cell formation in clusters.
5. Increased N:C ratio compared with normal hepatocytes.
6. Macroeosinophilic nucleoli.
7. Reduced number of binucleated cells.
8. Absence of epithelial cells.
9. Reticulin stain on cell block demonstrates reticulin loss and loss of the normal hepatic plate architecture (e.g. $\geq$ 3 cells thick).
10. Iron stain fails to stain tumour in cases of haemochromatosis.

Cytological appearance of high-grade hepatocellular carcinoma (moderate & poorly differentiated) :[19]

1. Peripherally wrapping endothelial smear pattern is virtually pathagnomonic.
2. Transgressing vessels are suggestive but cannot distinguish hepatocellular from renal cell carcinoma.
3. Presence of intracytoplasmic bile is pathagnomonic.
4. Polygonal cells with central nuclei and prominent nucleoli with visible, granular to clear cytoplasm in moderately differentiated tumors ; scant to no cytoplasm in poorly differentiated tumors.
5. Immunophenotype Low molecular weight cytokeratin, Polyclonal CEA , CDIO (canalicular) and HepPar-1 are positive. High MW cytokeratin (AE1) is usually negative.

The diagnosis of moderately differentiated hepatocellular carcinoma is usually straightforward because they look like normal liver while at the same time demonstrating obvious malignant features. At one end of spectrum, the tumor is well differentiated and it resembles liver and is difficult to distinguish from benign and reactive hepatocytic nodules. While at the other end of spectrum the tumor is poorly differentiated and is obviously malignant but may be difficult to appreciate its hepatic origin and to differentiate it from metastatic deposits in the liver.

Differentiation between well differentiated hepatocellular carcinoma and benign hepatic lesions :[27]

Benign hepatocytic nodules and mass lesions include

1. Macro regenerative nodule
2. Dysplastic nodule
3. Focal nodular hyperplasia
4. Hepatocellular adenoma

They are all discussed together because they share many cytologic features and distinction between them on cytology alone is not possible.

Cytologic features of Benign and Reactive Hepatocytic Nodules :[19]

1. Variably cellular smear (but not densely cellular).
2. No peripherally wrapping endothelial cells.
3. Clusters may have transgressing endothelial cells.
4. Mild pleomorphism of cell and nuclear size, sporadically placed large, atypical cells (dysplastic hepatocytes / large cell change).
5. Many binucleated hepatocytes.
6. Variably prominent nucleoli but no macro eosinophilic nucleoli.
7. Cytoplasm is generally abundant and granular but may show fatty change, lipofuscin pigment, or iron deposition.
8. Reticulin stain reveals retained framework of one or two cell layers on cell block.

One very helpful clue for well differentiated hepatocellular carcinoma is the presence of one of the two characteristic endothelial patterns.

1. **Basketing endothelial cells wrap groups or trabeculae. This pattern is seen in**

50% of hepatocellular carcinoma but is specific for hepatocellular carcinoma.

This pattern is seldom seen in benign hepatic lesions or other malignancies.

2. **Traversing capillaries through groups of hepatocytes. This pattern is noted in over 90% of hepatocellular carcinoma but is less specific than the "basketing" pattern since it can be seen in other malignancies and rarely, some non-neoplastic liver conditions.**

Acinar formation and the presence of prominent "cherry red" nucleoli favour well differentiated hepatocellular carcinoma. Ancillary studies may be helpful in differentiating benign and neoplastic hepatocytes. Decrease or absent reticulin staining or positive staining pattern outlining trabeculae greater than three cells thickness support the diagnosis of hepatocellular carcinoma.

Others have shown that the diffuse immunostaining with CD34 and factor VIII also favor hepatocellular carcinoma. Positive alpha - fetoprotein

staining is reported in 40% of hepatocellular carcinoma, but negative staining does not rule out a diagnosis of hepatocellular carcinoma [(19)].

Differentiation between high grade hepatocellular carcinoma and metastatic adenocarcinoma:[27]

In many instances, a known history of primary tumor is available and the task is to determine whether the morphology of the liver lesion is compatible with that of the known primary tumor. However, when a history is not available, the questions that need to be addressed will be " is it primary?" or "is it metastatic?". A markedly elevated serum alpha - fetoprotein level and the finding of a single lesion with or without satellite lesions on imaging favour a primary tumor over a metastatic disease.

Cytologically, bile production as evidenced by the presence of bile in the cytoplasm of malignant cells or in canaliculi between malignant cells, is considered diagnostic of hepatocellular carcinoma. Unfortunately bile is present in only half of the cases.

Although the "basketing" endothelial pattern is pathagnomonic for hepatocellular carcinoma, it is often absent in poorly differentiated turnours. The presence of "traversing" capillaries is less specific and can be seen in some metastatic lesions , particularly renal cell carcinoma. The key in diagnosing a poorly differentiated hepatocellular carcinoma is to look for

better differentiated cells with more typical hepatocytic features. Immunocytochemistry is of little help in differentiating poorly differentiated hepatocellular carcinoma from metastatic lesions because of a lack of highly specific markers.

Canalicular staining pattern with antibodies against polyclonal carcinoma embryonic antigen (pCEA) and diffuse positive staining with endothelial cell markers (such as CD 34, factor VIII) can help distinguishing hepatocellular carcinoma from metastatic adenocarcinoma. But positive staining with these markers is least often identified in poorly differentiated hepatocellular carcinoma.

Another relatively new marker HepPar I, has been shown to be quite specific and sensitive as a marker for hepatocellular carcinoma. About 83% to 100% of hepatocellular carcinoma stained positive with HepPar I but only 4% to 15% of metastatic carcinomas were positive. Unfortunately, only 56% of poorly differentiated hepatocellular carcinoma expressed HepPar 1[(27)].

ALPHA - FETOPROTEIN :

History:

Alpha - fetoprotein was first identified in human fetal sera by Bregstrand & Czar in 1956 on paper elecrophoregrams. Tatarinov demonstrated embryo-specific Alpha-globulin in the serum of patients with hepatocellular carcinoma .A few years later, it became clear that alpha - fetoprotein is a

valuable marker in the differential diagnosis of hepatocellular carcinoma. Moreover, it was found during clinical studies that alpha - fetoprotein is also associated with teratoblastoma of the testis and ovarian germ cell tumors.

Several highly sensitive methods of alpha - fetoprotein detection were elaborated in the early 1970's,but the most practical one was found to be Radio Immuno Assay (RIA) first introduced by Ruoshlati and Seppala. This assay permitted the detection of alpha - fetoprotein from its background level to its maximal values in different pathologies. Later, RIA was supplanted by enzyme-linked immunosorbent assay(ELISA) which is identical to RIA in specificity and sensitivity but more stable and ecologically acceptable.

Biology of alpha - fetoprotein ontogeny :

Alpha - fetoprotein is an embryo-specific alpha-globulin that is a major component of early embryonal serum in mammals. The amount of alpha - fetoprotein in embryonal serum is higher than the initial level of serum albumin, a predominant protein of late fetal and adult sera. The synthesis of alpha - fetoprotein is initiated with embryonal hematopoeisis in the yolk sac, particularly in the yolk sac visceral endoderm (YSVE).The YSVE is the site of synthesis of all serum proteins in the early embryonic development including serum albumin, alpha 1-antitrypsin, transferrin and early alpha - fetoprotein . Later, when embryonal hematopoeisis is replaced by fetal hematopoeisis, in which the liver becomes the main site of blood formation, the synthesis of alpha - fetoprotein and

the other serum proteins is transferred to the liver. Trace synthesis of alpha - fetoprotein is also noted in the fetal gut, as the YSVE, fetal gut and liver are closely related developmentally.

Alpha - fetoprotein is an obligatory component of embryonal and fetal hematopoeisis but little if anything is known regarding the nature of the association whether it is functional, includes common regulatory links, or is only coincidental[28].

Alpha - fetoprotein structure and function :

The structure of alpha - fetoprotein has been relatively well - characterised. It is a glycoprotein with a small (approximately 4%) carbohydrate moeity represented by one oligosaccharide residue. The protein moeity has been completely determined, consisting of one polypeptide chain of 590 amino acids arranged in three well defined domains, with a total molecular weight of approximately 69,000[29].

Regulation of Alpha - fetoprotein Expression at the Cellular Level:

The developmental regulation of alpha - fetoprotein synthesis and the reasons for its reappearance in malignancy are among the most avid areas of alpha - fetoprotein research. The first site of alpha - fetoprotein synthesis is the earliest differentiated embryonic structure, the YSVE. This was shown by immunohistochemical methods and by direct demonstration of alpha - fetoprotein synthesis in tissue culture. It is very important to note that even in this tissue alpha

- fetoprotein synthesis in not constitutive but regulated by intercellular interactions.

Alpha - fetoprotein is detected from the very onset of liver formation, produced by all hepatocytes in the liver bud, and continues to be expressed in mice and rats up to delivery and in human fetuses up to the second half of pregnancy. The decline of alpha - fetoprotein production coincides with the formation of the definitive liver plates, which are seen well in postnatal mice and rats. This decline begins in the portal area and moves in a gradient like manner to the central veins. The bright ring of alpha - fetoprotein positive cells persists around the central veins up to the complete cessation of its synthesis. It should be emphasised that hepatocyte proliferation per se is not responsible for alpha - fetoprotein synthesis, as alpha - fetoprotein synthesis may be resumed in non proliferating liver cells, as well as remain dormant in proliferating tissue.

The inhibition of alpha - fetoprotein synthesis appears reversible in all or at least most hepatocytes. It is demonstrated that alpha - fetoprotein synthesis in mature hepatocytes is reversibly repressed under the influence of intercellular interactions. The nature of this crucial interaction and the pathway from intercellular contact to specific gene activation currently await further study.

Because not all hepatocellular carcinoma produce alpha - fetoprotein , its presence is not essential to hepatocellular carcinogenesis and there is no evidence that tumours that do not produce alpha - fetoprotein are biologically different from the majority of hepatocellular carcinoma. Synthesis of alpha - fetoprotein by a tumour is permanent and age related .The younger the patient, the more likely the serum value is raised and the higher the level attained, provided that patients are age-matched. There is no sex difference in alpha - fetoprotein production. No obvious correlation exists between the serum concentration of alpha - fetoprotein and any of the clinical or biochemical features of the tumor or the survival time after diagnosis. However, small presymptomatic tumours are associated with an appreciably lower serum levels of alpha - fetoprotein than are symptomatic tumours. Attempts to correlate the degree of differentiation of hepatocellular carcinoma with the production of alpha - fetoprotein have produced conflicting results[30].

Because both false positive and false negative results are obtained when alpha - fetoprotein is used as a serum marker for hepatocellular carcinoma the research for an ideal tumour marker continues. A number of alternative markers have been suggested although none has proved to be more useful than alpha - fetoprotein [30].

Alpha - fetoprotein in hepatocellular carcinoma of varying differentiations :

With hepatocellular carcinoma, despite a relatively high incidence of increased alpha - fetoprotein levels of up to 70-80% over the background level, the correlation of increased alpha - fetoprotein levels of pathological grade is less clear.

1. The overall tendency is for resumed alpha - fetoprotein production in poorly differentiated tumours and for its absence in highly differentiated hepatocellular carcinoma. However, many exceptions exist, including loss of alpha - fetoprotein production by strongly alpha - fetoprotein positive tumour strains even without any visible changes in the degree of differentiation.
2. Large heterogeneity of alpha - fetoprotein content within an alpha - fetoprotein positive tumor, such that it is possible to isolate alpha - fetoprotein producing and non producing clones from the same tumor, demonstrating the reversibility of this trait.
3. In moderately differentiated hepatocellular carcinoma there is absence of correlation between cell morphology and alpha - fetoprotein production[28].

Clear and unequivocal interpretation of alpha - fetoprotein levels in hepatocellular carcinoma requires a knowledge of the precursor cells of hepatocellular carcinoma, as well as their alpha - fetoprotein status. The so called oval cells, discovered in rat liver at the acute phase of hepatocarcinogen action and alpha - fetoprotein production by these cells, became the first candidates for liver

stem cells or their close progeny, and the most plausible candidates for hepatocellular carcinoma precursor cells. Although this hypothesis helped to explain alpha - fetoprotein production by hepatocellular carcinoma , there is no evidence that a significant part of hepatocellular carcinoma is derived from the oval cells[28].

It is possible that the hepatocyte, and not only its precursor may serve as a progenitor of hepatocellular carcinoma. Further more, the state of the mature hepatocyte is determined by intercellular and cell-matrix interactions and hence is dependent on matrix composition and the presence of receptors responsible for such interactions. The developmental regulation of alpha - fetoprotein may be based on the appearance of putative receptors associated with the transition from hepatoblast to hepatocyte with simultaneous formation of adequate matrix. Poorly differentiated anaplastic hepatomas may be devoid of receptors, while highly differentiated tumours may have both systems unimpaired, leading to alpha - fetoprotein suppression. In moderately differentiated hepatocellular carcinoma, variations in matrix composition in a particular tumour and in different parts of the same tumor may in part explain the tremendous variation in alpha - fetoprotein production. Mutations and epigenetic events may further account for this variation. Clearly, much investigation remains in order to advance the understanding of alpha - fetoprotein production in hepatocellular carcinoma.

Alpha - fetoprotein in other conditions :

Hepatoblastoma is a distinct entity from hepatocellular carcinoma, consisting of hepatoblast like cells similar to embryonic liver parenchyma cells and secreting large amounts of alpha - fetoprotein in upto 90% of tumours[28].

In some 66% of men with testicular teratomas, elevated levels of alpha - fetoprotein are found and are usually associated with endodermal sinus tumour differentiation. Embryonal carcinomas can show alpha - fetoprotein positive cells, whereas pure seminomas, teratomas, and choriocarcinomas are negative for alpha - fetoprotein. Data suggest that there are two molecular variants of alpha - fetoprotein; Conconavalin A reactive and Conconavalin A nonreactive. The former is found predominantly in serum samples from patients with hepatocellular carcinoma and hepatoblastoma, and the latter is elevated in serum of patients with germ cell tumours[31].

Ovarian neoplasms that express alpha - fetoprotein include yolk sac tumours and tumours showing hepatoid differentiation, including hepatoid carcinoma and metastatic hepatocellular carcinoma. Among germ cell tumors, alpha - fetoprotein expression is almost entirely confined to yolk sac tumours, although focal expression can be seen in the rare embryonal carcinoma. Therefore alpha - fetoprotein expression in ovarian surface epithelial tumours is generally considered to be definitive support for a yolk sac tumor given the appropriate

morphologic context. This is particularly important in the distinction of true endometrioid proliferations from the endomertrioid yolk sac tumour[31].

Alpha - fetoprotein producing carcinomas, hepatoid or otherwise, are increasingly being recognized at extra hepatic sites. Aileen Wee and Anjula Thomas have reported two cases – one of gastric Adenocarcinoma with a liver mass whose hepatic aspiration revealed an alpha - fetoprotein producing Adenocarcinoma and the other was an endocervical adenocarcinoma with multiple liver nodules whose aspiration revealed an alpha - fetoprotein producing undifferentiated carcinoma[32].

Alpha - fetoprotein producing carcinomas occur in extrahepatic sites like the lung, gastrointestinal tract, female genital tract and urinary tract. Some of these tumours have hepatoid morphology, prompting one to search for a hepatocelluar carcinoma within the liver and to ascertain the serum alpha - fetoprotein levels and tumour immunoreactivity for alpha - fetoprotein[31]. Alpha - fetoprotein producing carcinomas represent about 5% of gastric cancers and of these, hepatoid adenocarcinoma is the most frequently observed histologic type[32].

Alpha - fetoprotein producing carcinomas of primary extra hepatic origin were also noted in cholangioma, angiofibromyxoma, oesophageal malignancy, gastric carcinoma, duodenal carcinoma, colorectal carcinoma, pancreatic carcinoma and cervical carcinoma[46].

SERUM ALPHA - FETOPROTEIN LEVELS :

A sensitive radio immunoassay for alpha - fetoprotein has been employed in the screening for primary hepatocellular carcinoma. About 70% to 90% of patients with hepatocellular carcinoma have elevations in serum alpha - fetoprotein and significant elevations are also observed in patients with germ cell tumours, other gastro intestinal malignancies and non – neoplastic hepatitis ,viral and alcoholic hepatitis and primary biliary cirrhosis. To enhance the specificity of this test in the diagnosis of hepatocellular carcinoma, a minimum concentration for postivity of 400 ng/ml has usually been assumed although this arbitrary cut off may exclude upto one third of patients with biopsy proven hepatocellular carcinoma[33].

A recently developed monoclonal radio immunoassay may improve the specificity of alpha - fetoprotein screenings. Levels of alpha - fetoprotein found in 10 – 12% of hepatocellular carcinoma cases are below the specificity threshold of the serum assay, as is the case for benign liver disease and liver metastases. For differentiating these tumour types, dynamic studies of alpha - fetoprotein level are essential. In this regard a stable or rising alpha - fetoprotein has been shown to be typical of hepatocellular carcinoma, an undulating pattern is characteristic of cirrhosis and a wave like pattern is suggestive of viral hepatitis.

In a study by Chen and Sung, in 60 cases of hepatocellular carcinoma, the tumor cell differentiation was classified according to Edmondson. Serum alpha - fetoprotein was detected with double diffusion in 3 out of 16 (19%) cases of the mature cell type (Grade I to II) and in 34 out of 44 cases (77%) of the immature cell type (Grade III to IV). The difference was statistically significant ($p < 0.025$)[46].

The most important advantages of alpha - fetoprotein measurement in surgery is in the detection of tumour recurrence. Serial quantitative determinations of serum alpha - fetoprotein provide important follow up information on hepatocellular carcinoma patients. A steady rise in serum alpha - fetoprotein usually implies a tumour recurrence[34].

Fucosylated alpha - fetoprotein is heterogeneous in structure and has a difference in the oligosaccharide side chain of alpha - fetoprotein. Several reports have attested the usefulness of fucosylated alpha - fetoprotein in differentiating hepatocellular carcinoma from benign hepatic parenchymal lesions. This refinement is particularly useful in the differential diagnosis of hepatocellular carcinoma when the serum alpha - fetoprotein concentration is less than 400 ng/ml and it may improve the diagnostic yield of alpha - fetoprotein in presymptomatic tumours. Unfortunately the method now used to measure fucosylated alpha - fetoprotein is rather complex and costly[30].

OTHER SEROLOGICAL TUMOR MARKERS IN HCC :[30]

Various tumour markers are being evaluated in the early detection of hepatocellular carcinoma. Their specificity and sensitivity varies.

	Sensitivity	Specificity
1. Des – gamma – carboxy Prothrombin	58 – 91%	84%
2. Alpha– L – Fucosidase	75%	70 – 90%
3. CA 125	High	Low
4. Tissue Polypeptide antigen	High	Low
5. Tumour associated isoenzymes of 5’ – nucleotide phosphodiseterase	High	Low
6. Tumour associated iso enzymes of gamma glutamyl transferase	Low	High
7. Variant Alkaline Phosphatase	Low	High
8. Ferritin	Low	Low
9. Carcino embryonic antigen	Low	Low
10. CA 19 – 9	Low	Low
11. Abnormal Vit B12 binding protein	Specific for fibro lamellar variant of hepatocellular carcinoma	
12. Neurotensin	Specific for fibro lamellar variant of hepatocellular carcinoma	

IMMUNOCYTOCHEMISTRY :

Immunocytochemistry has over the years evolved into a revolutionary diagnostic tool for the pathologist. The necessity for some special training method can be recognized from the fact that surgical pathology is a subjective discipline. Despite the presence of various diagnostic cytologic criteria, there is overlapping among different entities and dissimilarities among the same entity. This when compounded with subjective disparity among pathologists, reproducibility of diagnosis becomes difficult[35].

This prompted the development of special staining techniques to stain cells of particular lineage; marking the beginning of histochemistry in the mid nineteenth century. Francois Vincent Raspaid is the earliest botanist to use immunochemistry. Advent of aniline dyes revolutionized immunochemistry from 1862 to 1929.

With the development of immunology, a new method of staining was developed incorporating immunological techniques. Here, antibodies labeled with special stains are used to identify special antigens in the tissue. Combination of antibody with antigen gives a complex which imparts a particular colour to the cells with specific antigen.

Immunocytochemistry is an important adjunct method in cytologic diagnosis. The origin of immunocytochemistry techniques lies in the pioneering work of Albert Coons, starting in 1941. He described his first attempts to label antibodies directly with fluorescent isocyanate, antigens today are usually detected by the indirect technique introduced in 1955, that is, an unlabeled antibody is followed by a fluorescein or enzyme labeled second antibody.

The immunocytochemistry data have to be considered together with the other information available to the cytopathologist. Immunocytochemistry should never be selected as the sole or only method of diagnosis but must be integrated into the cytologic decision making process[36].

DETECTION SYSTEM :

Subsequent to the development of specific antibodies to the antigens, next step for the immunochemist was to de ? echniques to visualize the antigen antibody complex. In direct method the primary antibody is conjugated directly to the label. Most popular direct conjugates are those which are labeled with a fluorochrome, horse radish peroxidase and alkaline phosphates. The advantage of this method is that they are simple to use as they only require one application of reagent followed by appropriate chromogen substrate solution[37].

The sensitivity of immunochemical stains was significantly improved with the development of an indirect technique[38]. This is a two step method in which labeled secondary antibody reacts with the antigen bound primary antibody. Further increase in sensitivity over the indirect technique was achieved with the introduction of peroxide - anti peroxide enzyme complex. In this method, the secondary antibody serves as a linking antibody between the primary antibody and the peroxide - antiperoxide complex. Subsequent developments exploited the strong affinity of avidin for biotin and resulted in Avidin Biotin Complex method of H.S.U.et al[39]. This technique employs an enzyme labeled Avidin Biotin Complex which is mixed prior to use and forms a complex with a biotinylated

secondary antibody. Avidin Biotin Complex method increased reagent sensitivity when compared to the peroxide - antiperoxide reagent.

BIOTIN – STREPTAVIDIN SYSTEM :

Avidin Biotin complex (ABC) method is modified for substituting streptavidin for Avidin. In comparison to ABC method, Streptavidin Biotin method is four to eight times more sensitive[40]. Streptavidin, a tetrameric 60KD avidin analog isolated from the bacterium streptomyces avidinii is capable of binding biotin with a very high affinity. The use of streptavidin is preferred to avidin for several reasons.

1. Streptavidin contains no carbohydrates, which bind nonspecifically to lectin like substances found in normal tissue from kidney, liver, brain and mast cells.
2. The isoelectric point of streptavidin is close to neutrality, whereas avidin has an isoelectric point of 10; thus streptavidin conjugates do not exhibit the nonspecific electrostatic binding characteristic of avidin conjugates, which are positively charged under physiologic conditions.
3. Because the enzyme is directly conjugated to streptavidin in BSA system, it is a highly stable reagent that can be diluted and stored for long periods in a ready to use form[31].

CURRENT TECHNIQUES :

Enhanced polymer one step staining method (EPOS) is a new direct technique reported by Pluzek et al in 1993. In this method, a large number of primary antibody molecules and peroxidase enzymes are attached to a dextran polymer "back bone". This is rapid, can be used for frozen sections and sensitive enough to demonstrate small amounts of antigen.

Dextran polymer conjugate Two Step visualization system is a new indirect system based on dextran technology employed in the EPOS labeling system. The primary antibody in the EPOS model is replaced with a secondary antibody. This method offers greater sensitivity than the traditional indirect systems, is less time consuming than the three stage Avidin Biotin systems and does not read with endogenous biotin[37].

CURRENT APPLICATIONS OF IMMUNOCYTOCHEMISTRY :

1. **Analysis of tumour origin : Generally, tumours are classified histogenetically (such as epithelial, mesenchymal or neural) or by their origin (such as lung, breast, liver). Many antibodies recognise antigens that are expressed by cells of specific histogenesis. The antibodies with widest use are those that indicate the embryologic origin of cells.**
2. To predict the behavior of the tumour by identifying the prognostic markers.

3. Identification of Infections : The availability of antibodies against microbial agents and more recently, of nucleus acid probes targeting microbial DNA or RNA has bred the development of wide range of immunocytochemical techniques or in situ hybridisation techniques for the detection of specific types of organisms like HBsAg.[36]

ALPHA - FETOPROTEIN IN IMMUNOCYTOCHEMISTRY OF HEPATOCELLULAR CARCINOMA :

The finding of a hepatic tumour with immunoreactivity for alpha - fetoprotein is very suggestive of hepatocellular carcinomas, and its presence in poorly differentiated tumours may be of particular diagnostic utility(13)

In a study by C.Brumm and C.Schulze, alpha - fetoprotein was found in 16/63 (24%) of hepatocellular carcinomas and in two hepatoblastomas. When comparing tissue positivity for alpha - fetoprotein with tumour differentiation, grade1 hepatocellular carcinomas were found to be negative, while 21% of grade 2, 36% of grade 3 and 16% of grade 4, respectively stained positively. alpha - fetoprotein positive cells were present in 9/10 hepatocellular carcinomas with serum levels exceeding 5000 ng / ml, but were absent in 17 tumors with serum alpha - fetoprotein levels below 5000 ng / ml all tumours other than hepatocellular carcinomas and hepatoblastomas were alpha - fetoprotein negative[41].

According to Orlos, W.M.Bedrossian and Rosa.Davila alpha - fetoprotein was positive in four of eight hepatocellular carcinomas and concluded that alpha - fetoprotein has been considered a reliable marker of hepatic origin for a tumor arising in the liver, provided that a germ cell neoplasm has been excluded. alpha - fetoprotein was immunocytochemically negative in all 41 tumours of nonhepatic origin that were investigated[42].

In a study by Lee F.Fucich and Mary K. Cheles serum alpha - fetoprotein levels were elevated in four of seven patients with hepatocellular carcinoma and alpha - fetoprotein was expressed immunohistochemically in all four of these patients with hepatocellular carcinoma[43].

In a study by Ingebrog A. Koelma and Marius Nap, the presence of alpha - fetoprotein was noted in 3 cases out of 26 cases and the expression of alpha - fetoprotein could also be demonstrated in the series of alcoholic hepatitis (1 of 11) secondary biliary cirrhosis (2 of 10) and focal nodular hyperplasia (1 of 8). It was negative in 15 cases of metastases to liver[44].

In a study conducted by Swan N. Thung and Michael A.Gerber, alpha - fetoprotein was detected immunochemically in 11 cases (35%) among 37 hepatocellular carcinomas. Also Alpha – 1 antitrypsin was expressed in 73% of hepatocellular carcinomas. CEA was less common. HBsAg, but not HBcAg, was

observed in tumor cells in seven of nine hepatocellular carcinomas from HBsAg positive patients[45].

According to a study by Pui Chee Wu and Jane Wing Sang alpha - fetoprotein was detected in tumor tissue in 101 (40.9%) of the 254 patients tested. A higher proportion of poorly differentiated hepatocellular carcinomas expressed alpha - fetoprotein when compared to well differentiated Hepatocellular Carcinoma. Also HepPar1 reactive antigen was detected in 289 out of 290 patients (99.7%)[47].

In a study by Fumio Nomura & Kunihiko Ohnishi they compared the cytological features of hepatocellular carcinomas with serum alpha - fetoprotein levels. A total of 606 patients were divided into four groups based on their serum alpha - fetoprotein levels at the time of diagnosis.

Group 1 (< 20 ng/ml) N = 125

Group 2 (20 – 1000 ng/ml) N = 256

Group 3 (1000 – 10,000 ng/ml) N = 149

Group 4 (>10,000 ng/ml) N = 76

Increasing prevalence of group 1 patients and decreasing prevalence of group 4 were noted over a period of 9 years. And it was also noted that poorly differentiated tumours tend to be alpha - fetoprotein positive more often than well differentiated tumours[48].

In a study by Chan K. MA and Richard J.Zarbo, Alpha Feto – Protein was positive in 19% of hepatocellular carcinomas. This frequency is similar to that of most studies, and confirms lack of sensitivity of this antibody to alpha - fetoprotein. Yet it appears that alpha - fetoprotein is still a worthwhile diagnostic stain, because poorly differentiated hepatocellular carcinomas have a high probability of expressing alpha - fetoprotein than well differentiated hepatocellular carcinomas [49].

Alpha - fetoprotein disappears from the blood after birth but reappears in 80% to 90% of patients with hepatocellular carcinomas. However, it can be demonstrated by immunocytochemistry in only 12% to 62% of hepatocellular carcinoma. An alpha - fetoprotein staining is focal and most often found in the undifferentiated neoplasms[50]. Alpha - fetoprotein is a reasonably specific marker for hepatocellular carcinomas. It has been noted to be absent in small well differentiated hepatocellular carcinomas [36].

Other immunocytochemical markers in hepatocellular carcinomas [13] :

I. Greatest Diagnostic Utility

S.NO	FEATURES	SENSITIVITY	*Comments*
1	p CEA (Canalicular staining)	50 – 90 %	Near 100% specificity; beware trapped non – neoplastic hepatocytes and mimics of canalicular pattern in non – HCC; often negative in poorly differentiated HCC
2	m-CEA (non canalicular Staining)	0 – 10%	Rarely positive in HCC, 60 – 75% of cholangiocarcinoma metastatic Adenocarcinoma positive.
S.No	**FEATURES**	**Sensitivity**	***Comments***
3	Hep Par – 1	80%	90% specificity, beware trapped non neoplastic hepatocytes rarely cholangiocarcinoma or metastatic Adeno carcinoma positive.
4	ERY – 1	90%	95% specificity, may be found in renal cell carcinoma, yolk sac tumour, transitional cell carcinoma; staining often focal; normal hepatocyte is positive.

II. Some diagnostic utility[13]

S.NO	FEATURES	SENSITIVITY	*Comments*
1	Hepatocyte CK (8, 18) versus other cytokeratins	94 – 100 Verus 30 – 60	Lack of specificity, but of use if only "hepatocyte CK" positive.
2	CD 34 (endothelium)	50 – 100	Rare positive in cirrhotic liver : Prominent in advanced HCC, 50% of small well differentiated hepatocellular carcinomas are negative

III. Least diagnostic utility and / or few data[13]

S.NO	FEATURES	SENSITIVITY	*Comments*
1	∝ 1 Microglobulin	95	Near 90% specificity; need more data
2	Albumin	Nearly 100	Frequent false – positive results, prominent background staining
3	Inhibin	5 – 90	Higher rate appears to be false – positive, biotin not blocked
4	PTHrP	0	All CC positive, metastatic adenocarcinoma may be positive (best frozen tissue)
5	A1- AT	55 – 93	Lack of specificity and / or sensitivity

6	EMA	40	Lack of specificity and / or sensitivity
7	B72, 3	5 – 10	Lack of specificity and / or sensitivity
8	Ber – EP4	35	Lack of specificity and / or sensitivity

S.NO	**FEATURES**	**SENSITIVITY**	***Comments***
9	HMFG – Z	20	Lack of specificity and / or sensitivity
10	Cu – 18	10	Lack of specificity and / or sensitivity
11	TPA	30 (Weak)	Lack of specificity and / or sensitivity
12	Lau – M1 / CD 15	5 – 30	Lack of specificity and / or sensitivity
13	Ferritin	45 – 70	Lack of specificity and / or sensitivity
14	Factor XIII a	65 – 70	Lack of specificity and / or sensitivity
15	Synaptophysin	5 – 10	Focal positivity does not exclude HCC versus neuro endocrine tumour.
16	Chromogranin	5	Focal positivity does not exclude HCC versus neuro – endocrine tumour

Immunocytochemistry using unabsorbed polyclonal anti CEA anti serum or certain monoclonal CEA (m – CEA) antibodies, each of which cross reacts with canalicular biliary glycoprotein 1, demonstrates bile canaliculi (canalicular pattern) in 70% to 80% of hepatocellular carcinomas. (range 24% to 90%)[50]

Canalicular CEA staining remains one of the most useful and most thoroughly investigated immunohistochemical markers in the differential diagnosis of hepatocellular carcinomas, although one drawback is that this pattern of immunoreactivity is most frequently seen in better differentiated tumours. About 50% of poorly differentiated tumours lack immunoreactivity. A false positive hepatocellular carcinoma interpretation of a canalicular pattern may result from inclusion of immunoreactive non - neoplastic hepatocytes within the tumour, misinterpretation of an incomplete membrane pattern as canalicular in location, or misinterpretation of periluminal immunoreactivity in adeno carcinomas as staining of dilated canaliculi. cytoplasmic immunoreactivity with m – CEA antisera is uncommon, and for this reason, absence of immunoreactivity with this antibody may be diagnostically useful[50].

Hep Par – 1 is a relatively hepatocyte specific monoclonal antibody that reacts with a hepatocyte epitope. Its staining pattern suggests organelle localization, possibly mitochondrial studies from University of Pittsburgh in Pennsylvania have shown performance characteristics similar to those of p – CEA with 82% sensitivity and 90% specificity. HepPar – 1 has been shown to be useful for distinguishing hepatocellular carcinoma from cholangiocarcinoma and

metastatic adeno carcinoma in most settings[51] although positivity is occasionally found in cholangio carcinoma. HepPar – 1 is probably best used as part of a panel of immuno markers[52].

With the availability of an array of markers the problem is in choosing one marker wisely. However in practice, many investigators currently use a panel of p – CEA (canalicular pattern), m – CEA, HepPar – 1 and alpha - fetoprotein antibodies when evaluating diagnostically challenging cases[13].

MATERIALS AND METHODS

Cases diagnosed as hepatocellular carcinoma on fine needle aspiration smears received in the Department of Cytology, PSG IMS & R during a period from December 2003 to December 2004 were taken. The slides were stained with Leishman's and Papanicolou stain. These were graded as low grade and high grade as per the guidelines given by Martha Bishop Pitman in Diagnostic Cytology of Liver Aspirates in Robert. D.Odze's – Surgical Pathology of the G.I.Iract, Liver, Biliary tract & Pancreas, First Edition.

The unstained slides fixed in alcohol were used for running immunocytochemistry by using Streptavidin Biotin Complex technique.

Method :

Streptavidin Biotin Complex technique.

Antibody :

Primary antibody – polyclonal rabbit Anti Human alpha - fetoprotein supplied from DAKOCYTOMATION

PRINCIPLE OF STREPTAVIDIN BIOTIN COMPLEX TECHNIQUE :

The high affinity of glycoprotein for biotin was harnessed in this technique. Originally the egg white protein avidin was used as a source of glycoprotein rich element. But the presence of oligosaccharides in the molecule made it non – specific. Hence streptavidin obtained from the culture broth of the bacterium streptomyces avidinii was used in the place of avidin. Though studies have come giving almost equal results in both streptavidin and avidin in general, streptavidin is the preferred protein. Biotin has the ability to conjugate multiple antibodies and enzyme markers. Hence biotinylated enzymes like peroxidase is bound to streptavidin which forms the streptavidin biotin complex. This complex can now attach to large number of antibodies, thus increasing the sensitivity.

REAGENTS USED :

1. **3% hydrogen peroxide in water**
2. **Primary Antibody – polyclonal rabbit anti human alpha - fetoprotein supplied from DAKO CYTOMATION.**
3. **TRIS buffer pH 7.4**
4. **DAKO labeled streptavidin biotin, horse radish peroxidase (DAKO LSAB 2 system, HRP)**
5. **Harris Haematoxylin**

6. DPX Mountant

PREPARATION OF TRIS BUFFER PH 7.4 :

1. **Preparation of stock solution A (0.2 m tris) Dissolve 2.42 grams TRIS (Mol. Wt 121) in 100 ml of distilled water.**
2. **Preparation of stock solution B (0.2 m HCL) 1.7 ml of HCL (Mol. wt. 36.46) mixed in 100 ml of distilled water.**
3. **Mix 30ml of solution A and 22.2 ml of solution B and made upto 100ml.**

IMMUNOCYTOCHEMISTRY WAS RUN AS FOLLOWS :

1. **The smears were placed in TRIS buffer pH 7.4 for 5 minutes.**
2. **Placed in 3% hydrogen peroxide for 10 minutes.**
3. **Washed in TRIS buffer for 5 minutes.**
4. **Then the slides were incubated with the primary antibody, polyclonal rabbit anti human alpha 1 - fetoprotein for 30 minutes.**
5. **Washed in TRIS buffer for 5 minutes**
6. **Biotinylated secondary antibody was applied to smears for 30 minutes**
7. **Washed in TRIS buffer for 5 minutes**
8. **Slides were incubated in streptavidin biotin complex linked horse radish peroxidase (DAKO, LSAB, Peroxidase, DAB) for 30 minutes.**
9. **Washed in TRIS buffer for 5 minutes**
10. **DAB was used as a chromogen for 30 minutes.**
11. **Washed thoroughly in running tap water.**
12. **Smears were counterstained with Harris haemotoxylin**

13. Smears were air dried and mounted with DPX.

Tumour cells were scored positive if there was golden brown cytoplasmic staining in the neoplastic cells. The presence or absence of nuclear staining was also noted. The expression of the marker was graded as follows

Grade 0 = no positive cells

Grade 1 = < 1% to 24% positive

Grade 2 = 25% to 49% positive

Grade 3 = 50% to 74% positive

Grade 4 = >75% positive cells in accordance to a study by Pui Chee Wu and Jane Wing Sang in American Journal of Pathology[53].

OBSERVATIONS

A total of 1,337 fine needle aspiration smears were reported in the Department of Pathology over a period of 1 year from December 2003 to December 2004. Out of these, 86 cases were guided fine needle aspirates of liver and 37 cases were reported as hepatocellular carcinoma. This worked out to an incidence of 42% among guided liver aspirates. The male : female ratio is as given in the Table I and Bar chart

TABLE I

Age and sex incidence of hepatocellular carcinoma with its grade

	Male			Female		
Age in years	**Low Grade**	**High Grade**	**Total**	**Low Grade**	**High Grade**	**Total**
< 40	-	-	**-**	1	-	**1**
40 - 59	2	2	**4**	-	-	**-**
60 - 69	3	5	**8**	2	1	**3**
≥ 70	2	5	**7**	1	1	**2**
Total			**19**			**6**

From this table, we infer that out of 25 cases, 19 were males and 6 were females and the male : female ratio is 4 : 1 approximately. The peak age incidence of hepatocellular Carcinoma in PSGIMS & R in both male and females was around 60 – 70 years and only a single case was observed in a

female aged 24 years. Out of this 25 cases, 11 were low grade (fig :1) and 14 were high grade (fig 2, 3 and 4).

HBsAg was positive in 2 cases out of 18 cases, amounting to 11% incidence. 7 cases were not evaluated for HBsAg.

Table II

HCV Status in hepatocellular carcinoma

Table II and Pie chart analyses the incidence of HCV infection in these 25 cases and it was found that 17 cases were positive for HCV and 8 were negative. Hence the incidence of HCV infection in hepatocellular carcinoma is 68%.

Table III

Correlation of serum alpha - fetoprotein level with hepatocellular carcinoma

Table IV

Correlation of serum alpha - fetoprotein with Low Grade and High Grade hepatocellular carcinoma

Table III, Table IV, pie chart and the line graph shows correlation of serum alpha - fetoprotein level with the grade of hepatocellular carcinoma. The serum alpha - fetoprotein was above 400 ng/ml in 12 cases., out of which, 6 cases had values above 1000 ng/ml. It was also observed that out of these 12 cases with serum alpha - fetoprotein level more than 400ng/ml ,11 cases turned out to be high grade hepatocellular carcinomas and 1 case was low grade hepatocellular carcinoma. .Thus, high grade hepatocellular carcinomas had high levels of serum alpha - fetoprotein (more than 400 ng/ml)

13 cases had serum alpha - fetoprotein level less than 400 ng/ml. Of these 13 cases, 5 cases had values between 11.5 ng/ml and 400 ng/ml and 8 cases had values between 5.1 ng/ml and 11.5ng/ml which is the normal value. Out of 8 cases which had normal serum alpha - fetoprotein level, 7 were low grade hepatocellular carcinoma and 1 was high grade hepatocellular

carcinoma. Among the 5 cases which had values ranging between 11.5 ng/ml and 400 ng/ml, 3 were low grade hepatocellular carcinoma and 2 were high grade hepatocellular carcinoma. The correlation of serum alpha - fetoprotein level with the grade of hepatocellular carcinoma was analysed with Chi-square Distribution table.

The significance of the X^2 Chi-square statistics is tested using a special distribution known as the Chi-square distribution. The calculated value of Chi-square is 11.97. The critical value given in the Chi-square table at 1% Level of Significance is 6.63 with one degree of freedom, that is Chi-square ,

X^2 Square

0.1 = 6.63

Chi – Square> Chi – Square 0.01

We conclude that the grade of hepatocellular carcinoma is related to serum alpha - fetoprotein value. Thus low grade hepatocellular carcinomas tend to have low levels of serum alpha - fetoprotein levels (less than 400 ng/ml) and high grade hepatocellular carcinomas have high levels of serum alpha - fetoprotein (more than 400 ng/ml)

This study also shows that 8 cases of cytologically proven hepatocellular carcinomas had normal serum alpha - fetoprotein levels and 7 of these were low grade hepatocellular carcinomas and 1 case was high grade hepatocellular carcinoma . In other words, low grade hepatocellular carcinomas may or may not express high serum levels of alpha - fetoprotein.

The Immunocytochemical analysis run on our slides is as given in Table V, VI line graph and bar diagram. The control for cellular alpha – fetoprotein expression was fetal liver(fig 5).

Table V
Percentage positivity of alpha – fetoprotein expression in varying differentiation of Hepatocellular Carcinoma

Table VI

Alpha - fetoprotein expression in low grade and high grade Hepatocellular carcinoma

Table V shows immunocytochemical expression of Grade O to Grade 2 positivity in low grade hepatocellular carcinoma and Grade 3 and Grade 4 positivity in high grade hepatocellular carcinoma .

It is observed in table VI that 12 cases had less than 50% cells expressing alpha - fetoprotein and 11 were low grade hepatocellular carcinoma (fig 6) and 1 was high grade hepatocellular carcinoma. 13 cases had more than 50% cells expressing alpha - fetoprotein and all of these were high grade hepatocellular carcinomas(fig 7,8). Thus, we understand that cytological expression of alpha - fetoprotein is high (more than 50%) in high grade tumours and low (less than 50%)in low grade tumours. Thus, immunocytochemical expression of alpha - fetoprotein is directly proportional to the grade of tumour.

DISCUSSION

Hepatocellular carcinoma is a very common malignancy in all African countries, south of Sahara and in South East Asia and is more common in males. Its association with viral hepatitis especially hepatitis C virus and hepatitis B virus has been well established . Serum alpha - fetoprotein levels remains the most useful marker for hepatocellular carcinoma (positive in 70% of cases) and levels above 400 ng/ml are indicative of malignancy and above 1000 ng/ml are definite for hepatocellular carcinoma[33]. It was also noted that immunocytochemical expression of alpha – fetoprotein in aspiration samples was directly proportional to the grade of hepatocellular carcinoma.

In our study it was observed that males were commonly affected than females and the male: female ratio was 4:1. The peak age incidence for both sexes was between 60 to 70 years. Only one case was observed in a young female aged 24 years.

Ikeda et al, after a fifteen year observation period reported the development rate of hepatocellular carcinoma as 75% for HCV and 25% for HBV infection. The current study shows similar trends. 68% of hepatocellular carcinomas were HCV positive while 11% of hepatocellular carcinomas were HBV positive. The declining incidence of HBV associated hepatocellular carcinoma could be due to the mass vaccination programmes.

The normal serum alpha – feto protein level in adults ranges from 5.1 to 11.5 ng/ml. Values greater than 10 ng/ml are noted in most of the diseases of the liver. Ding and Juei studied serum alpha – fetoprotein levels in various liver diseases[46]. They observed values between 10 to 400 ng/ml in most of the non-neoplastic lesions (34% in cirrhosis, 58% in chronic hepatitis and in 3 out of 13 cases in healthy HbsAg carriers, etc.,)

They also observed that serum alpha - fetoprotein was greater than 400 ng/ml in 86 out of 125 cases (69%) of hepatocellular carcinoma.

In our study, the serum alpha - fetoprotein was above 400 ng/ml in 12 cases(48%). Out of these, 11 cases were high grade hepatocellular carcinoma and 1 was low grade hepatocellular carcinoma.

The serum alpha - fetoprotein was below 400ng/ml in 13 cases, out of which 10 cases were low grade hepatocellular carcinoma and 3 were high grade hepatocellular carcinoma. On testing the significance of association between grade of hepatocellular carcinoma and serum alpha - fetoprotein level, it was observed that the critical value given in the Chi – square table at 1% Level of Significance is 6.63 with One degree of freedom. Thus, it can be concluded that low grade hepatocellular carcinoma tend to have low serum alpha - fetoprotein values (<400 ng/ml) and high grade hepatocellular carcinomas had high serum alpha - fetoprotein values (>400 ng/ml).

In a study by Fumio Nomura & Kunihiko Ohnishi they compared the cytological features of hepatocellular carcinoma with serum alpha - fetoprotein levels. A total of 606 patients were divided into four groups based on their serum alpha – fetoprotein levels at the time of diagnosis.

Group 1 (< 20 ng/ml) N = 125

Group 2 (20 – 1000 ng/ml) N = 256

Group 3 (1000 – 10,000 ng/ml) N = 149

Group 4 (>10,000 ng/ml) N = 76

Increasing prevalence of group 1 patients and decreasing prevalence of group 4 were noted over a period of 9 years. And it was also noted that poorly differentiated tumours tend to be alpha – fetoprotein positive more often than well – differentiated tumours.

C.Brumm and C.Schulze on comparing tissue positivity for alpha - fetoprotein with tumour differentiation, they found that,

Grade 1	-	27% Negative
Grade 2	-	21% Positive
Grade 3	-	36% Positive
Grade 4	-	16% Positive

Alpha - fetoprotein positive cells were present in 9/10 hepatocellular carcinoma with serum levels exceeding 5000 ng/ml but were absent in 17 tumours with serum alpha - fetoprotein levels below 5000 ng/ml[41].

According to a study by Pui Chee Wu and Jane Wing Sang alpha - fetoprotein expression was graded as follows ,

Grade O - No Positive Cells

Grade 1 - <1 to 24% Positive

Grade 2 - 25 to 49% Positive

Grade 3 - 50 to 74% Positive

Grade 4 - ≥ 75% Positive cells

Alpha – fetoprotein was detected in tumor cells in 101 patients (40.9%) of the 254 patients tested. A higher proportion of poorly differentiated hepatocellular carcinoma expressed alpha - fetoprotein when compared to well differentiated hepatocellular carcinoma. Also HepPar 1 reactive antigen was detected in 289 out of 290 patients (99.7%)[47].

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In a study by Chan K. MA and Richard J.Zarbo, alpha - fetoprotein was positive in 19% of hepatocellular carcinomas. This frequency is similar to that of most studies, and confirms lack of sensitivity of this antibody to alpha - fetoprotein. Yet it appears that alpha - fetoprotein is still a worthwhile

diagnostic stain, because poorly differentiated hepatocellular carcinomas have a high probability of expressing alpha - fetoprotein than well differentiated hepatocellular carcinomas [49].

In our study, immunocytochemistry run on the alcohol fixed slides showed that alpha - fetoprotein expression was less than 50% in 12 cases, out of which 11 cases (91%) were low grade hepatocellular carcinoma and 1 case (9%) was high grade hepatocellular carcinomas. Thus, in low grade hepatocellular carcinomas the alpha - fetoprotein expression was found to be less than 50%. On the other hand, all the 13 cases (100%) that had more than 50% of cells expressing alpha - fetoprotein were high grade hepatocellular carcinomas. Thus, high grade hepatocellular carcinoma had higher percentage of expression of alpha - fetoprotein . Hence, it was observed that immunocytochemical expression of alpha - fetoprotein was directly proportional to the grade of differentiation of hepatocellular carcinomas.

CLINICAL, SEROLOGICAL FINDINGS AND CYTOLOGICAL EXPRESSION IN THE PATIENTS STUDIED

Characteristics	Data
Number of Patients	25
Male : Female	4 :1
Mean Age (Range)	65 (60 to 70)
HBsAg Positive	11%
HCV Positive	68%
Differentiation a) Low Grade b) High Grade	 11 14

Characteristics	Data
Serum alpha - fetoprotein	
1. Less than 400 ng/ml	
Total	13 Cases
(a) Low Grade	10 Cases
(b) High Grade	3 Cases
2. More than 400 ng/ml	
Total	12 Cases
(a) Low Grade	1 Case
(b) High Grade	11 Cases
Cytological Expression of alpha - fetoprotein	
1. Number of Positive Cases	22 Cases (88%)
2. Less than 50% Expression	12 Cases
(a) Low Grade	11 Cases
(b) High Grade	1 Case
3. More than 50% Expression	13 Cases
(a) Low Grade	0
(b) High Grade	13 Cases

SUMMARY AND CONCLUSION

Hepatocellular carcinoma is one of the most common malignancies and is a leading cause of cancer related death in developing countries. The incidence of hepatocellular carcinoma was found to be 42% among guided liver aspirates at PSG IMS & R.

The purpose of this study was to determine whether alpha - fetoprotein could be used to aid in the diagnosis of hepatocellular Carcinoma and to correlate the percentage positivity of cytological alpha - fetoprotein expression with the differentiation of hepatocellular carcinoma. Also the serological factors such as serum alpha - fetoprotein level, HBsAg and HCV status were correlated. To evaluate the above factors, 25 cases of cytologically diagnosed hepatocellular carcinomas were chosen and the slides were stained using Streptavidin Biotin complex method with DAB as chromogen. The cytological alpha - fetoprotein expression was then studied.

It was observed that alpha - fetoprotein was expressed cytologically in 88%(22 cases) of hepatocellular carcinomas. Thus alpha - fetoprotein staining is

helpful if positive, but a negative stain in no way rules out the presence of a

tumour. Also all the 3 tumours that were negative for alpha - fetoprotein staining were cytologically low grade hepatocellular carcinomas. Thus low grade tumours tend to be negative for alpha - fetoprotein.

The percentage of cells expressing alpha - fetoprotein increased in relation to the grade of differentiation. All the low grade hepatocellular carcinomas (100%) which had positive staining showed less than 50 % of the cells to be positive and 92 % of high grade hepatocellular carcinomas that had positive staining showed more than 50% of the cells to be positive. Thus it was observed that the expression of alpha - fetoprotein was directly proportional to the grade of tumour, i.e., high grade tumours show higher percentage of expression.

The cytological grading also correlated well with serum alpha - fetoprotein level as per Chi – Square distribution table concluding that high grade hepatocellular carcinoma had higher serum alpha - fetoprotein value (more than 400 ng/ml) and low grade hepatocellular carcinoma has lower serum alpha – fetoprotein value (less than 400 ng / ml).

The incidence of HBsAg and HCV infection was found to be 11% and 68% respectively in the hepatocellular carcinomas.

CONCLUSIONS

1. **Alpha - fetoprotein is a reliable immunocytochemical marker for hepatocellular carcinoma.**
2. **The expression of alpha - fetoprotein in the cells was found to be directly proportional to the grade of hepatocellular carcinoma.**

Further studies with more number of cases can be done to prove that this might be a useful marker to differentiate between low grade and high grade hepatocellular carcinoma cases.

Also, alpha - fetoprotein can be included among a panel of other known markers like HepPar 1, p – CEA and m– CEA in diagnosis of hepatocellular carcinoma and the contribution by each of these markers in all cases can be evaluated and diagnostic accuracy can be enhanced.

3. **Serum alpha - fetoprotein level is also related to the grade of hepatocellular carcinoma. Levels more than 400 ng/ml are common in high grade hepatocellular carcinoma.**
4. **HCV is more often associated with hepatocellular carcinoma (68%) than HBV (11%).**

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